UK Intellectual Property Office

For Creativity and Innovation

Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

he undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of Comptroller-General, hereby certify that annexed hereto is a true copy of the documents originally filed in connection with patent application GB0118153.6 filed on 25 July 2001.

ccordance with the Patents (Companies Re-registration) Rules 1982, if a company named is certificate and any accompanying documents has re-registered under the Companies 1980 with the same name as that with which it was registered immediately before re-tration save for the substitution as, or inclusion as, the last part of the name of the words lic limited company" or their equivalents in Welsh, references to the name of the any in this certificate and any accompanying documents shall be treated as references to me with which it is so re-registered.

ordance with the rules, the words "public limited company" may be replaced by p.l.c., L.C. or PLC.

stration under the Companies Act does not constitute a new legal entity but merely s the company to certain additional company law rules.

Signed J Tomkins

Dated 7 February 2008

Patents Form 1/77

Patents Act 1977
(Rule 16)

1/77

26JUL01 E648027-10 D02246
P01/7700 0.00-0118153.6

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

THE PATENT OFFICE
M
25 JUL 2001
LONDON

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

P011073GB CLM

2. P
(

0118153.6

25 JUL 2001

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

LORANTIS LIMITED
Babraham Hall
Babraham
Cambridge CB2 4UL

Patents ADP number *(if you know it)*

7911845001

If the applicant is a corporate body, give the country/state of its incorporation

GB

4. Title of the invention

A METHOD

5. Name of your agent *(if you have one)*

D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

21 New Fetter Lane
London
EC4A 1DA

Patents ADP number *(if you know it)*

59006

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	47 ✓
Claim(s)	4 ✓
Abstract	1 ✓
Drawing(s)	3 + 3 ✓

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	1 ✓
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature D Young & Co (Agents for the Applicants) Date 25 July 2001

12. Name and daytime telephone number of person to contact in the United Kingdom: Catherine Mallalieu 020 7353 4343 ~~023 8071 9500~~

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

A METHOD

Field of the invention

The present invention relates to a method of detecting modulators of Notch signalling. The present invention also relates to novel modulators identifiable by such a method and uses thereof in therapy. The present invention also relates to a pharmaceutical composition comprising at least one such modulator.

Background of the Invention

Notch signal transduction plays a critical role in cell fate determination in vertebrate and invertebrate tissues. Notch is expressed at many stages of Drosophila embryonic and larval development and in many different cells implying a wide range of functions including an important role in neurogenesis and in the differentiation of mesodermal and endodermal cells. There are at least four mammalian Notch genes (Notch-1, Notch-2, Notch-3 and Notch-4). Notch-1, which most closely resembles the proteins of invertebrates and lower vertebrates, is widely expressed and is essential for early development. Recent evidence suggests that Notch signalling contributes to lineage commitment of immature T-cells in the thymus.

During maturation in the thymus, T-cells acquire the ability to distinguish self-antigens from those that are non-self, a process termed "self tolerance". Tolerance to a non-self antigen, however, may be induced by immunisation under specific conditions with a peptide fragment comprising that antigen. In autoimmune diseases such as multiple sclerosis, rheumatoid arthritis or diabetes, there is a failure of the proper regulation of tolerance. Improved treatment methods for re-establishing tolerance are desirable for autoimmune diseases. Similarly in allergic conditions and for transplantation of an organ or tissue from a donor individual, induction of tolerance to particular foreign antigens or profiles of foreign antigens is desirable.

The expression on the cell surface of normal adult cells of the peripheral immune system of Notch and its ligands, Delta and Serrate, suggests a role for these proteins in T-cell acquired immunocompetence. T-cells express Notch-1 mRNA constitutively. Delta expression is limited to only a subset of T-cells in the peripheral lymphoid tissues. Serrate expression is restricted to a subset of antigen presenting cells (APCs). These observations reinforce the view that the Notch receptor ligand family continues to regulate cell fate decisions in the immune system beyond embryonic development with Notch signalling playing a central role in the induction of peripheral unresponsiveness (tolerance or anergy), linked suppression and infectious tolerance (Hoyne et al.).

Thus, as described in WO 98/20142, manipulation of the Notch signalling pathway can be used in immunotherapy and in the prevention and/or treatment of T-cell mediated diseases. In particular, allergy, autoimmunity, graft rejection, tumour induced aberrations to the T-cell system and infectious diseases caused, for example, by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas, Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara, may be targeted.

It has also recently been shown that it is possible to generate a class of regulatory T cells which are able to transmit antigen-specific tolerance to other T cells, a process termed infectious tolerance (WO98/20142). The functional activity of these cells can be mimicked by over-expression of a Notch ligand protein on their cell surfaces or on the surface of antigen presenting cells. In particular, regulatory T cells can be generated by over-expression of a member of the Delta or Serrate family of Notch ligand proteins. Delta or Serrate induced T cells specific to one antigenic epitope are also able to transfer tolerance to T cells recognising other epitopes on the same or related antigens, a phenomenon termed "epitope spreading".

Notch ligand expression also plays a role in cancer. Indeed, upregulated Notch ligand expression has been observed in some tumour cells. These tumour cells are capable of rendering T cells unresponsive to restimulation with a specific antigen, thus providing a possible explanation of how tumour cells prevent normal T cell responses. By

downregulating Notch signalling *in vivo* in T cells, it may be possible to prevent tumour cells from inducing immunotolerance in those T cells that recognise tumour-specific antigens. In turn, this would allow the T cells to mount an immune response against the tumour cells (WO00/135990).

There remains a need in the art for the provision of further diagnostic or therapeutic compositions useful in the detection, prevention and treatment of diseases or conditions of, or relating to, the immune system, and in particular, but not exclusively, T cell mediated diseases or disorders. The present invention addresses this problem by delivering an effective method of identifying novel modulators of the Notch signalling pathway. While many assay methods are known in the art, the present invention is based in our knowledge of the Notch signalling pathway and realisation that an effective assay method for detection of novel modulators needs to be carried out using a cell of the immune system.

Statements of the Invention

According to one aspect of the present invention there is provided a method for detecting modulators of Notch signalling comprising the step of monitoring Notch signalling in a cell of the immune system in the presence and absence of a candidate modulator and determining whether the candidate modulator modulates Notch signalling.

According to another aspect of the present invention there is provided a method for detecting modulators of Notch signalling comprising the steps of:

(a) contacting a cell of the immune system with a candidate modulator;

(b) monitoring Notch signalling; and

(c) determining whether the candidate modulator modulates Notch signalling.

The candidate modulator may be any organic or inorganic compound. Preferably the candidate modulator is selected from a group consisting of: small natural or synthetic

molecule compounds, a polypeptide, a polynucleotide, an antibody or a fragment of an antibody and a cytokine or a fragment of a cytokine.

In a preferred embodiment, the step of monitoring Notch signalling comprises the steps of monitoring levels of expression of at least one target gene. The target gene may be an endogenous target gene of the Notch signalling pathway or a reporter gene.

Known endogenous target genes of the Notch signalling pathway include Deltex, Hes-1, E(spl), Il-10, CD-23, Dlx-1, CTLA4, CD-4, Numb, Mastermind and Dsh.

Many reporter genes are standard in the art and include genes encoding an enzymatic activity, genes comprising a radiolabel or a fluorescent label and genes encoding a predetermined polypeptide epitope.

Preferably at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling. Even more preferably, at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling and a second signal, and/or a third signal wherein the second and third signals are different.

An example of a signal of use in the present invention is a signal that results from activation of a signalling pathway specific to cells of the immune system, such as a T cell receptor (TCR) signalling pathway, a B cell receptor (BCR) signalling pathway or a Toll-like receptor (TLR) signalling pathway, with or without an accessory signal (known in the art as costimulatory signals for T and B cell receptor signalling).

Another example of a signal of use in the present invention is a costimulus specific to cells of the immune system such as B7 proteins including B7.1-CD80, B7.2-CD86, B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD28, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134, CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205,

CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLR) such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FCγ receptor 2 (CD32), CD64 (FCγ receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors or growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.

In a preferred embodiment, the method of the present invention is carried out in a T cell or T cell progenitor or an antigen presenting cell (APC). APCs are cells which are capable of expressing MHC class II molecules and able to present antigens to CD4+ T cells. Preferably, the APC will be a myeloid lineage cell such as a dendritic cell, for example a Langerhan cell, a monocyte or macrophage or a primary cell or a B lineage cell.

Levels of expression of at least one target gene can be monitored with a protein or a nucleic acid assay.

According to a further aspect of the present invention there is provided a modulator identifiable by the method of the invention.

According to another aspect of the present invention there is provided the use of a modulator according to the present invention in the preparation of a medicament for the treatment of a disease or condition of, or related to the immune system. Preferably, the disease is a T-cell mediated disease.

According to yet another aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of at least one modulator according to the invention and a pharmaceutically acceptable carrier, diluent and/or excipient.

Detailed Description

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples and with reference to the accompanying drawings in which:

Figure 1 and figure 2 show schematic representations of the Notch signalling pathway;

Figure 3 shows a schematic representation of Notch and examples of immune cell signalling pathways which may be used in screening for immune cell modulators of Notch signalling.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general texts is herein incorporated by reference.

The present invention relates to an assay method for detecting modulators of Notch signalling.

Notch Signalling

As used herein, the expression "Notch signalling" is synonymous with the expression "the Notch signalling pathway" and refers to any one or more of the upstream or downstream events that result in, or from, (and including) activation of the Notch receptor.

Notch signalling directs binary cell fate decisions in the embryo. Notch was first described in *Drosophila* as a transmembrane protein that functions as a receptor for two different ligands, Delta and Serrate. Vertebrates express multiple Notch receptors and ligands. At least four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) have been identified to date in human cells.

Notch proteins are synthesized as single polypeptide precursors that undergo cleavage via a Furin-like convertase that yields two polypeptide chains that are further processed to form the mature receptor. The Notch receptor present in the plasma membrane comprises a heterodimer of two Notch proteolytic cleavage products, one comprising an N-terminal fragment consisting of a portion of the extracellular domain, the transmembrane domain and the intracellular domain, and the other comprising the majority of the extracellular domain. The proteolytic cleavage step of Notch to activate the receptor occurs and is mediated by a furin-like convertase.

Notch receptors are inserted into the membrane as disulphide-linked heterodimeric molecules consisting of an extracellular domain containing up to 36 epidermal growth factor (EGF)-like repeats and a transmembrane subunit that contains the cytoplasmic domain. The cytoplasmic domain of Notch contains six ankyrin-like repeats, a polyglutamine stretch (OPA) and a PEST sequence. A further domain termed RAM23 lies proximal to the ankyrin repeats and, like the ankyrin-like repeats, is involved in binding to a transcription factor, known as Suppressor of Hairless [Su(H)] in *Drosophila* and CBF1 in vertebrates (Tamura). The Notch ligands also display multiple EGF-like repeats in their extracellular domains together with a cysteine-rich DSL (Delta-Serrate Lag2) domain that is characteristic of all Notch ligands (Artavanis-Tsakonas).

The Notch receptor is activated by binding of extracellular ligands, such as Delta, Serrate and Scabrous, to the EGF-like repeats of Notch's extracellular domain. Delta requires cleavage for activation. It is cleaved by the ADAM disintegrin metalloprotease Kuzbanian at the cell surface, the cleavage event releasing a soluble and active form of Delta. An oncogenic variant of the human Notch-1 protein, also known as TAN-1, which has a truncated extracellular domain, is constitutively active and has been found to be involved in T-cell lymphoblastic leukemias.

The cdc10/ankyrin intracellular-domain repeats mediate physical interaction with intracellular signal transduction proteins. Most notably, the cdc10/ankyrin repeats interact with Suppressor of Hairless [Su(H)]. Su(H) is the *Drosophila* homologue of C-promoter binding factor-1 [CBF-1], a mammalian DNA binding protein involved in the Epstein-Barr virus-induced immortalization of B-cells. It has been demonstrated that, at least in cultured cells, Su(H) associates with the cdc10/ankyrin repeats in the cytoplasm and translocates into the nucleus upon the interaction of the Notch receptor with its ligand Delta on adjacent cells. Su(H) includes responsive elements found in the promoters of several genes and has been found to be a critical downstream protein in the Notch signalling pathway. The involvement of Su(H) in transcription is thought to be modulated by Hairless.

The intracellular domain of Notch (NotchIC) also has a direct nuclear function (Lieber). Recent studies have indeed shown that Notch activation requires that the six cdc10/ankyrin repeats of the Notch intracellular domain reach the nucleus and participate in transcriptional activation. The site of proteolytic cleavage on the intracellular tail of Notch has been identified between gly1743 and val1744 (termed site 3, or S3) (Schroeter). It is thought that the proteolytic cleavage step that releases the NotchIC for nuclear entry is dependent on Presenilin activity.

The intracellular domain has been shown to accumulate in the nucleus where it forms a transcriptional activator complex with the CSL family protein CBF1 (suppressor of

hairless, Su(H) in *Drosophila*, Lag-2 in *C. elegans*) (Schroeter; Struhl). The NotchIC-CBF1 complexes then activate target genes, such as the bHLH proteins HES (hairy-enhancer of split like) 1 and 5 (Weinmaster). This nuclear function of Notch has also been shown for the mammalian Notch homologue (Lu).

NotchIC processing occurs only in response to binding of Notch ligands Delta or Serrate/Jagged. The post-translational modification of the nascent Notch receptor in the Golgi (Munro; Ju) appears, at least in part, to control which of the two types of ligand it interacts with on a cell surface. The Notch receptor is modified on its extracellular domain by Fringe, a glycosyl transferase enzyme that binds to the Notch/Lin motif. Fringe modifies Notch by adding *O*-linked fucose groups to the EGF-like repeats (Moloney; Bruckner). This modification by Fringe does not prevent ligand binding, but may influence ligand induced conformational changes in Notch. Furthermore, recent studies suggest that the action of Fringe modifies Notch to prevent it from interacting functionally with Serrate/Jagged ligands but allow it to preferentially interact with Delta (Panin; Hicks). Although *Drosophila* has a single Fringe gene, vertebrates are known to express multiple genes (Radical, Manic and Lunatic Fringes) (Irvine).

Thus, signal transduction from the Notch receptor can occur via different pathways (Figures 1-3). The better defined pathway involves proteolytic cleavage of the intracellular domain of Notch (NotchIC) that translocates to the nucleus and forms a transcriptional activator complex with the CSL family protein CBF1 (supressor of hairless, Su(H) in *Drosophila*, Lag-2 in *C. elegans*). NotchIC-CBF1 complexes then activate target genes, such as the bHLH proteins HES (hairy-enhancer of split like) 1 and 5. Notch can also signal in a CBF1-independent manner that involves the cytoplasmic zinc finger containing protein Deltex (Figure 3). Unlike CBF1, Deltex does not move to the nucleus following Notch activation but instead can interact with Grb2 and modulate the Ras-Jnk signalling pathway.

As described above, several endogenous modulators of Notch are already known. These include, for example, the Notch ligands Delta and Serrate. An aim of the present invention is the detection of novel Notch signalling modulators.

Candidate Modulators

The term "modulate" as used herein refers to a change or alteration in the biological activity of the Notch signalling pathway or a target signalling pathway thereof. The term "modulator" may refer to antagonists or inhibitors of Notch signalling, i.e. compounds which block, at least to some extent, the normal biological activity of the Notch signalling pathway. Conveniently such compounds may be referred to herein as inhibitors or antagonists. Alternatively, the term "modulator" may refer to agonists of Notch signalling, i.e. compounds which stimulate or upregulate, at least to some extent, the normal biological activity of the Notch signalling pathway. Conveniently such compounds may be referred to as upregulators or agonists.

The candidate modulator of the present invention may be an organic compound or other chemical. In this embodiment, the candidate modulator will be an organic compound comprising two or more hydrocarbyl groups. Here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen. The candidate modulator may comprise at least one cyclic group. The cyclic group may be a polycyclic group, such as a non-fused polycyclic group. For some applications, the agent comprises at least the one of said cyclic groups linked to another hydrocarbyl group.

In one preferred embodiment, the candidate compound will be an amino acid sequence or a chemical derivative thereof, or a combination thereof. In another preferred embodiment, the candidate compound will be a nucleotide sequence - which may be a sense sequence or an anti-sense sequence. The candidate modulator may also be an antibody.

Candidate modulators may be synthetic compounds or natural isolated compounds. Various examples of such synthetic or natural modulators are listed below.

Candidate modulators: antagonists

Antagonists of Notch signalling will include any molecule which is capable of inhibiting Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway.

Candidate modulators for Notch signalling inhibition may be dominant negative versions of a compound capable of activating or transducing Notch signalling. Alternatively, the candidate modulator of Notch signalling will be capable of repressing a compound capable of activating or transducing Notch signalling. In a further alternative embodiment, the modulator will be an inhibitor of Notch signalling.

In a particular embodiment, the modulator will be capable of reducing or preventing Notch or Notch ligand expression. Such a modulator may be a nucleic acid sequence capable of reducing or preventing Notch or Notch ligand expression. Endogenous such modulators include nucleic acid sequences encoding a polypeptide selected from Toll-like receptor protein family, a cytokine such as IL-12, IFN-γ, TNF-α, or a growth factor such as a bone morphogenetic protein (BMP), a BMP receptor and activins. Candidate modulators will include derivatives, fragments, variants, mimetics, analogues and homologues of any of the above.

In a preferred embodiment, the modulator will be a polypeptide, or a polynucleotide encoding such a polypeptide, that decreases or interferes with the production of

compounds that are capable of producing an increase in the expression of Notch ligand. Endogenous compounds of this type include Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors. Candidate modulators will include derivatives, fragments, variants, mimetics, analogues and homologues of any of the above.

Alternatively, the candidate modulator will be an antisense construct derived from a sense nucleotide sequence encoding a polypeptide selected from a Notch ligand and a polypeptide capable of up-regulating Notch ligand expression, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants, mimetics, analogues and homologues thereof.

In another preferred embodiment the candidate modulator for Notch signalling inhibition will be a molecule which is capable of modulating Notch-Notch ligand interactions. A molecule may be considered to modulate Notch-Notch ligand interactions if it is capable of inhibiting the interaction of Notch with its ligands, preferably to an extent sufficient to provide therapeutic efficacy. In this embodiment the modulator may be a polypeptide, or a polynucleotide encoding such a polypeptide, selected from a Toll-like receptor, a cytokine such as IL-12, IFN-γ, TNF-α, or a growth factor such as a BMP, a BMP receptor and activins, derivatives, fragments, variants, mimetics, homologues and analogues thereof. Preferably the modulator will decrease or interfere with the production of an agent that is capable of producing an increase in the expression of Notch ligand, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants, mimetics homologues and analogues thereof.

Preferably when the modulator is a receptor or a nucleic acid sequence encoding a receptor, the receptor is activated. Thus, for example, when the modulator is a nucleic acid sequence, the receptor is constitutively active when expressed.

Modulators for Notch signalling inhibition also include downstream modulators of the Notch signalling pathway (such as Dsh, Numb and derivatives, fragments, variants, mimetics, homologues and analogues thereof), compounds that prevent expression of Notch target genes or induce expression of genes repressed by the Notch signalling

pathway and dominant negative versions of Notch signalling transducer molecules (such as of NotchIC, Deltex and derivatives, fragments, variants, mimetics, homologues and analogues thereof). Proteins for Notch signalling inhibition will also include variants of the wild-type components of the Notch signalling pathway which have been modified in such a way that their presence blocks rather than transduces the signalling pathway. An example of such a modulator would be a Notch receptor which has been modified such that proteolytic cleavage of its intracellular domain is no longer possible.

Candidate modulators: agonists

Agonists of Notch signalling will include any molecule which is capable of up-regulating Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway. Candidate modulators for up-regulating the Notch signalling pathway include compounds capable of transducing or activating the Notch signalling pathway.

Modulators for Notch signalling transduction will include molecules which participate in signalling through Notch receptors including activation of Notch, the downstream events of the Notch signalling pathway, transcriptional regulation of downstream target genes and other non-transcriptional downstream events (e.g. post-translational modification of existing proteins). More particularly, such modulators will allow activation of target genes of the Notch signalling pathway.

According to one aspect of the present invention the modulator may be the Notch polypeptide or polynucleotide or a fragment, variant, derivative, mimetic or homologue thereof which retains the signalling transduction ability of Notch or an analogue of Notch which has the signalling transduction ability of Notch. By Notch, we mean Notch-1, Notch-2, Notch-3, Notch-4 and any other Notch homologues or analogues. Analogues of Notch include proteins from the Epstein Barr virus (EBV), such as EBNA2, BARF0 or LMP2A. In a particularly preferred embodiment the

modulator may be the Notch intracellular domain (Notch IC) or a sub-fragment, variant, derivative, mimetic, analogue or homologue thereof.

Modulators for Notch signalling activation include molecules which are capable of activating Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway.

Such a modulator may be a dominant negative version of a Notch signalling repressor. In an alternative embodiment, the modulator will be capable of inhibiting a Notch signalling repressor. In a further alternative embodiment, the modulator for Notch signalling activation will be a positive activator of Notch signalling.

In a particular embodiment, the modulator will be capable of inducing or increasing Notch or Notch ligand expression. Such a molecule may be a nucleic acid sequence capable of inducing or increasing Notch or Notch ligand expression.

In one embodiment, the modulator will be capable of up-regulating expression of the endogenous genes encoding Notch or Notch ligands in target cells. In particular, the modulator may be an immunosuppressive cytokine capable of up-regulating the expression of endogenous Notch or Notch ligands in target cells, or a polynucleotide which encodes such a cytokine. Immunosuppressive cytokines include IL-4, IL-10, IL-13, TGF-β and FLT3 ligand. Candidate modulators will therefore further include fragments, derivatives, variants, mimetics, analogues and homologues of any of the above.

Endogenous agonists include Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors. Candidate modulators may therefore include derivatives, fragments, variants, mimetics, analogues and homologues thereof, or a polynucleotide encoding any one or more of the above.

In another embodiment, the modulator may be a Notch ligand, or a polynucleotide encoding a Notch ligand. Notch ligands will typically be capable of binding to a Notch

receptor polypeptide present in the membrane of a variety of mammalian cells, for example hemapoietic stem cells. Endogenous Notch ligands include polypeptides of the Delta family, for example Delta-1 (Genbank Accession No. AF003522 - *Homo sapiens*), Delta-3 (Genbank Accession No. AF084576 - *Rattus norvegicus*), Delta-like 3 (*Mus musculus*), Delta-4 (Genbank Accession No. AB043894) and polypeptides of the Serrate family, for example Serrate-1 and Serrate-2 (WO97/01571, WO96/27610 and WO92/19734), Jagged-1 and Jagged-2 (Genbank Accession No. AF029778 - *Homo sapiens*), and LAG-2. Candidate compounds of the present invention include fragments, derivatives, variants, mimetics, analogues and homologues of any of the above.

In a preferred embodiment, the modulator will be a constitutively active Notch receptor or Notch intracellular domain, or a polynucleotide encoding such a receptor or intracellular domain.

In an alternative embodiment, the modulator of Notch signalling will act downstream of the Notch receptor. Thus, for example, the activator of Notch signalling may be a constitutively active Deltex polypeptide or a polynucleotide encoding such a polypeptide. Other endogenous downstream components of the Notch signalling pathway include Deltex-1, Deltex-2, Deltex-3, Suppressor of Deltex (SuDx), Numb and isoforms thereof, Numb associated Kinase (NAK), Notchless, Dishevelled (Dsh), emb5, Fringe genes (such as Radical, Lunatic and Manic), PON, LNX, Disabled, Numblike, Nur77, NFkB2, Mirror, Warthog, Engrailed-1 and Engrailed-2, Lip-1 and homologues thereof, the polypeptides involved in the Ras/MAPK cascade modulated by Deltex, polypeptides involved in the proteolytic cleavage of Notch such as Presenilin and polypeptides involved in the transcriptional regulation of Notch target genes. Candidate modulators of use in the present invention will therefore include constitutively active forms of any of the above, analogues, homologues, derivatives, variants, mimetics and fragments thereof.

Modulators for Notch signalling activation may also include any polypeptides expressed as a result of Notch activation and any polypeptides involved in the

expression of such polypeptides, or polynucleotides encoding for such polypeptides.

Activation of Notch signalling may also be achieved by repressing inhibitors of the Notch signalling pathway. As such, candidate modulators will include molecules capable of repressing any Notch signalling inhibitors. Preferably the molecule will be a polypeptide, or a polynucleotide encoding such a polypeptide, that decreases or interferes with the production or activity of compounds that are capable of producing an decrease in the expression or activity of Notch, Notch ligands, or any downstream components of the Notch signalling pathway. In a preferred embodiment, the modulators will be capable of repressing polypeptides of the Toll-like receptor protein family, cytokines such as IL-12, IFN-γ, TNF-α, and growth factors such as the bone morphogenetic protein (BMP), BMP receptors and activins.

Preferably, the modulator of the present invention will be a polypeptide or a polynucleotide.

Polypeptide Sequences

As used herein, the term "polypeptide" is synonymous with the term "amino acid sequence" and/or the term "protein". In some instances, the term "polypeptide" is synonymous with the term "peptide".

"Peptide" usually refers to a short amino acid sequence that is 10 to 40 amino acids long, preferably 10 to 35 amino acids.

The polypeptide sequence may be prepared and isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

Polynucleotide Sequences

As used herein, the term "polynucleotide sequence" is synonymous with the term "polynucleotide" and/or the term "nucleotide sequence".

The polynucleotide sequence may be DNA or RNA of genomic or synthetic or of recombinant origin. They may also be cloned by standard techniques. The polynucleotide sequence may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length and up to 1,000 bases or even more. Longer polynucleotide sequences will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction (PCR) under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

The nucleic acid may be RNA or DNA and is preferably DNA. Where it is RNA, manipulations may be performed via cDNA intermediates. Generally, a nucleic acid sequence encoding the first region will be prepared and suitable restriction sites provided at the 5' and/or 3' ends. Conveniently the sequence is manipulated in a standard laboratory vector, such as a plasmid vector based on pBR322 or pUC19 (see below). Reference may be made to Molecular Cloning by Sambrook *et al.* (Cold Spring Harbor, 1989) or similar standard reference books for exact details of the appropriate techniques.

Sources of nucleic acid may be ascertained by reference to published literature or databanks such as GenBank. Nucleic acid encoding the desired first or second sequences may be obtained from academic or commercial sources where such sources

are willing to provide the material or by synthesising or cloning the appropriate sequence where only the sequence data are available. Generally this may be done by reference to literature sources which describe the cloning of the gene in question.

Alternatively, where limited sequence data is available or where it is desired to express a nucleic acid homologous or otherwise related to a known nucleic acid, exemplary nucleic acids can be characterised as those nucleotide sequences which hybridise to the nucleic acid sequences known in the art.

The polynucleotide sequence may comprise, for example, a protein-encoding domain, an antisense sequence or a functional motif such as a protein-binding domain and includes variants, derivatives, analogues and fragments thereof. The term also refers to polypeptides encoded by the nucleotide sequence.

Variants, Derivatives, Analogues, Homologues and Fragments

In addition to the specific polypeptide and polynucleotide sequences mentioned herein, the present invention also encompasses the use of variants, derivatives, analogues, homologues, mimetics and fragments thereof.

In the context of the present invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues) has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be modified by addition, deletion, substitution modification replacement and/or variation of at least one residue present in the naturally-occurring protein.

The term "derivative" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of and/or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide retains at least one of its endogenous functions.

The term "analogue" as used herein, in relation to polypeptides or polynucleotides, includes any polypeptide or polynucleotide which retains at least one of the functions of the endogenous polypeptide or polynucleotide but generally has a different evolutionary origin thereto.

The term "mimetic" as used herein, in relation to polypeptides or polynucleotides, refers to a chemical compound that possesses at least one of the endogenous functions of the polypeptide or polynucleotide which it mimics.

Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required transport activity or ability to modulate Notch signalling. Amino acid substitutions may include the use of non-naturally occurring analogues.

Proteins of use in the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the transport or modulation function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

As used herein, the term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulfide bonds. The terms subunit and domain may also refer to polypeptides and peptides having biological function.

"Fragments" are also variants and the term typically refers to a selected region of the polypeptide or polynucleotide that is of interest either functionally or, for example, in an assay. "Fragment" thus refers to an amino acid or nucleic acid sequence that is a portion of a full-length polypeptide or polynucleodtide.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous

standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

Polynucleotide variants will preferably comprise codon optimised sequences. Codon optimisation is known in the art as a method of enhancing RNA stability and therefor gene expression. The redundancy of the genetic code means that several different codons may encode the same amino-acid. For example, Leucine, Arginine and Serine are each encoded by six different codons. Different organisms show preferences in their use of the different codons. Viruses such as HIV, for instance, use a large number of rare codons. By changing a nucleotide sequence such that rare codons are replaced by the corresponding commonly used mammalian codons, increased expression of the sequences in mammalian target cells can be achieved. Codon usage tables are known in the art for mammalian cells, as well as for a variety of other organisms. Preferably, at least part of the sequence is codon optimised. Even more preferably, the sequence is codon optimised in its entirety.

As used herein, the term "homology" can be equated with "identity". An homologous sequence will be taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical. In particular, homology should typically be considered with respect to those regions of the sequence (such as amino acids at positions 51, 56 and 57) known to be essential for an activity. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

Percent homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a

time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefor firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA (Atschul) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching. However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Nucleotide sequences which are homologous to or variants of sequences of use in the present invention can be obtained in a number of ways, for example by probing DNA libraries made from a range of sources. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the reference nucleotide sequence under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the amino acid and/or nucleotide sequences useful in the present invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of use in the present invention. Conserved sequences can be predicted, for example, by aligning the amino

acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used. The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such nucleotide sequences may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the nucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the activity of the polynucleotide or encoded polypeptide.

In a first step of the method of the present invention, any one or more of the above candidate modulators is brought into contact with a cell of the immune system. Cells of the immune system of use in the present invention are described below.

Cells of the Immune System

Cells of use in the present invention are cells of the immune system capable of transducing the Notch signalling pathway.

Most preferably the cells of use in the present invention are T-cells. These include, but are not limited to, $CD4^+$ and $CD8^+$ mature T cells, immature T cells of periferal or thymic origin and NK-T cells.

Alternatively, the cells will be antigen-presenting cells (APCs). APCs include dendritic cells (DCs) such as interdigitating DCs or follicular DCs, Langerhans cells, PBMCs, macrophages, B-lymphocytes, T-lymphocytes, or other cell types such as epithelial cells, fibroblasts or endothelial cells, constitutively expressing or activated to express a MHC Class II molecules on their surfaces. Precursors of APCs include $CD34^+$ cells, monocytes, fibroblasts and endothelial cells. The APCs or precursors

may be modified by the culture conditions or may be genetically modified, for instance by transfection of one or more genes.

The T cells or APCs may be isolated from a patient, or from a donor individual or another individual. The cells are preferably mammalian cells such as human or mouse cells. Preferably the cells are of human origin. The APC or precursor APC may be provided by a cell proliferating in culture such as an established cell line or a primary cell culture. Examples include hybridoma cell lines, L-cells and human fibroblasts such as MRC-5. Preferred cell lines for use in the present invention include Jurkat, H9, CEM and EL4 T-cells; long-term T-cell clones such as human HA1.7 or mouse D10 cells; T-cell hybridomas such as DO11.10 cells; macrophage-like cells such as U937 or THP1 cells; B-cell lines such as EBV-transformed cells such as Raji, A20 and M1 cells.

Dendritic cells (DCs) can be isolated/prepared by a number of means, for example they can either be purified directly from peripheral blood, or generated from $CD34^+$ precursor cells for example after mobilisation into peripheral blood by treatment with GM-CSF, or directly from bone marrow. From peripheral blood, adherent precursors can be treated with a GM-CSF/IL-4 mixture (Inaba *et al*), or from bone marrow, non-adherent $CD34^+$ cells can be treated with GM-CSF and TNF-α (Caux *et al*). DCs can also be routinely prepared from the peripheral blood of human volunteers, similarly to the method of Sallusto and Lanzavecchia (1994) using purified peripheral blood mononucleocytes (PBMCs) and treating 2 hour adherent cells with GM-CSF and IL-4. If required, these may be depleted of $CD19^+$ B cells and $CD3^+$, $CD2^+$ T cells using magnetic beads (Coffin *et al*). Culture conditions may include other cytokines such as GM-CSF or IL-4 for the maintenance and, or activity of the dendritic cells or other antigen presenting cells.

T cells and B cells for use in the invention are preferably obtained from cell lines such as lymphoma or leukemia cell lines, T cell hybridomas or B cell hybridomas but may also be isolated from an individual suffering from a disease of the immune system or a recipient for a transplant operation or from a related or unrelated donor individual. T

cells and B cells may be obtained from blood or another source (such as lymph nodes, spleen, or bone marrow) and may be enriched or purified by standard procedures. Alternatively whole blood may be used or leukocyte enriched blood or purified white blood cells as a source of T cells and other cell types. It is particularly preferred to use helper T cells ($CD4^+$). Alternatively other T cells such as $CD8^+$ cells may be used.

Candidate modulators of use in the present invention are brought into contact with a cell of the immune system as described above. In a further step, modulation of Notch signalling by a candidate modulator is detected. Assays for detecting modulation of Notch signalling will be described below. Many of these assays will involve monitoring the expression of a "target gene".

Target Genes

The target genes of use in the present invention may be endogenous target genes (i.e. endogenous target genes of the Notch signalling pathway) or synthetic reporter genes.

Endogenous target genes

Endogenous target genes of the Notch signalling pathway include Deltex, genes of the Hes family (Hes-1 in particular), Enhancer of Split [E(spl)] complex genes, Il-10, CD-23, Dlx-1, CTLA4, CD-4, Dll-1, Numb, Mastermind and Dsh. Although all genes the expression of which is modulated by Notch activation may be used for the purpose of the present invention, preferred endogenous target genes are described below.

Deltex, an intracellular docking protein, replaces Su(H) as it leaves its site of interaction with the intracellular tail of Notch, as shown in Figure 1. Deltex is a cytoplasmic protein containing a zinc-finger (Artavanis-Tsakonas; Osborne). It interacts with the ankyrin repeats of the Notch intracellular domain. Studies indicate that Deltex promotes Notch pathway activation by interacting with Grb2 and modulating the Ras-JNK signalling pathway (Matsuno). Deltex also acts as a docking protein which prevents Su(H) from binding to the intracellular tail of Notch (Matsuno). Thus, Su(H) is released into the

nucleus where it acts as a transcriptional modulator. Recent evidence also suggests that, in a vertebrate B-cell system, Deltex, rather than the Su(H) homologue CBF1, is responsible for inhibiting E47 function (Ordentlich). Expression of Deltex is upregulated as a result of Notch activation in a positive feedback loop. The sequence of Homo sapiens Deltex (DTX1) mRNA may be found in GenBank Accession No. AF053700.

Hes-1 (Hairy-enhancer of Split-1) (Takebayashi) is a transcriptional factor with a basic helix-loop-helix structure. It binds to an important functional site in the CD4 silencer leading to repression of CD4 gene expression. Thus, Hes-1 is strongly involved in the determination of T-cell fate. Other genes from the Hes family include Hes-5 (mammalian Enhancer of Split homologue), the expression of which is also upregulated by Notch activation, and Hes-3. Expression of Hes-1 is upregulated as a result of Notch activation. The sequence of human Hes-1 can be found in GenBank Accession Nos. AK000415 and AF264785.

The E(spl) gene complex [E(spl)-C] (Leimeister) comprises seven genes of which only E(spl) and Groucho show visible phenotypes when mutant. E(spl) was named after its ability to enhance Split mutations, Split being another name for Notch. Indeed, E(spl)-C genes repress Delta through regulation of achaete-scute complex gene expression. Expression of E(spl) is upregulated as a result of Notch activation.

IL-10 (interleukin-10) is a factor produced by Th2 helper T-cells. It is a co-regulator of mast cell growth and shows extensive homology with the Epstein-Barr bcrfi gene. Although it is not known to be a direct downstream target of the Notch signalling pathway, its expression has been found to be strongly upregulated coincident with Notch activation. The mRNA sequence of IL-10 may be found in GenBank ref. No. GI1041812.

CD-23 is the human leukocyte differentiation antigen CD23 (FCE2) which is a key molecule for B-cell activation and growth. It is the low-affinity receptor for IgE.

Furthermore, the truncated molecule can be secreted, then functioning as a potent mitogenic growth factor. Although it is not thought to be a direct downstream target of the Notch signalling pathway, its expression has been found to be strongly upregulated coincident with Notch activation. The sequence for CD-23 may be found in GenBank ref. No. GI1783344.

Dlx-1 (distalless-1) expression is downregulated as a result of Notch activation. Sequences for Dlx genes may be found in GenBank Accession Nos. U51000-3.

CTLA4 (cytotoxic T-lymphocyte activated protein 4) is an accessory molecule found on the surface of T-cells which is thought to play a role in the regulation of airway inflammatory cell recruitment and T-helper cell differentiation after allergen inhalation. The promoter region of the gene encoding CTLA4 has CBF1 response elements and its expression is upregulated as a result of Notch activation. The sequence of CTLA4 can be found in GenBank Accession No. L15006.

CD-4 expression is downregulated as a result of Notch activation. A sequence for the CD-4 antigen may be found in GenBank Accession No. XM006966.

Synthetic Reporter Genes

In an alternative embodiment of the present invention, the target gene is a reporter gene. In a preferred embodiment, the reporter gene is under the transcriptional control of a promoter region or responder element(s) sensitive to Notch signalling.

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Other protocols include enzyme-linked immunosorbent assay (ELISA),

radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes may even be used. These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al.

One skilled in the art will recognize that the identity of the specific reporter gene can, of course, vary. Examples of reporter genes that have been used in the art include, but are not limited to, genes encoding an enzymatic activity such as chloramphenicol acetyltransferase (CAT) gene, Green Fluorescent Protein (GFP), luciferase (luc), ß-galactosidase, invertase, horseradish peroxidase, glucuronidase, exo-glucanase, glucoamylase or alkaline phosphatase. Alternatively, the reporter gene may comprise a radiolabel or a fluorescent label such as FITC, rhodamine, lanthanide phosphors, or a green fluorescent fusion protein (See for example Stauber et al). Alternatively, the reporter may comprise a predetermined polypeptide epitope which can be recognized by a secondary reporter such as leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, or epitope tags. One skilled in the art will appreciate that the specific reporter gene or genes utilized in the methods disclosed herein may vary and may also depend on the specific model system utilized, and the methods disclosed herein are not limited to any specific reporter gene or genes.

By way of further examples, a number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for assay procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241.

The reporter gene used in the method of the present invention is under the transcriptional control of at least one Notch signalling sensitive promoter region and/or

responder element. Promoter regions and/or responder elements sensitive to Notch signalling include the regulatory elements of endogenous Notch target genes such as the HES promoters, Deltex promoter, Notch and Notch ligand promoters, IL-10 promoters. Regulatory elements of use in the present invention also include single or multimerized CBF1 sites, CTLA4 promoters and AIRE promoters. The regulatory elements are positioned such that activation of the Notch signalling pathway results in increased expression of the reporter gene.

One or more copies of the reporter gene can be inserted into the host cell by methods known in the art. The term "host cell" - in relation to the present invention includes any cell that could comprise the target for the agent of the present invention. Polynucleotides may be introduced into prokaryotic cells or eukaryotic cells, for example yeast, insect or mammalian cells. Preferably, the host cell will be a cell of the immune system as described above.

Polynucleotides of the invention may be introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

In the present invention, the host cells will preferably be mammalian cells and the polypeptides will be expressed either intracellularly, on the cell membranes or secreted in a culture media if preceded by an appropriate leader sequence.

Expression of the target genes (whether endogenous or synthetic reporter genes) may be dependent on Notch signalling alone or on Notch signalling and one or more further stimulatory signals.

Stimulatory Signals

Expression or repression of the target genes (endogenous or reporter genes) of use in the present invention is dependent on Notch signalling. In a preferred embodiment, expression or repression of the target genes will additionally be depend on a second immune cell specific stimulus, with or without an accessory signal (or "costimulus").

In one embodiment, the second stimulus will result from activation of an immune cell receptor. Examples of immune cell receptors include T cell receptors (TCR), B cell receptors (BCR) and Toll-like receptors (TLR). Examples of molecules capable of triggering a TCR or BCR signal include specific antigens for the receptors, superantigens such as TSS1, SEA, SEB, SEC, SED and SEE, antibodies to the TCR αβ chains including Fab, F(ab)2 fragments, phage displayed peptides and ScFV or antibodies to CD3 proteins including ζ and ϵ chains, anti-BCR antibodies, LPS and other bacterial products, cell receptors involved in phagocytosis such as Fc receptors, complement receptors, mannose receptors and other scavenger receptors, receptors involved in clearance of apoptotic cells such as CD36 and αvβ5, dendritic cell receptors such as DEC205 and DC-light, and activators of TCR and/or BCR signalling pathways such as PMA, ionomycin or kinase inhibitors. These molecules may be used alone or in combination and may be presented on an anticgen presenting cell.

Accessory or costimulatory signals of immune cell receptor signalling include B7 proteins such as B7.1-CD80, B7.2-CD86, B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD28, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134, CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205, CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLRs), such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FCγ receptor 2 (CD32), CD64 (FCγ receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors and growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.

In one embodiment, the second stimulus will be a costimulus. In an alternative embodiment, expression of the target genes will depend on three separate stimuli: Notch signalling, immune cell signalling and a costimulus, all of which are described above. The signals may be delivered all at once or may be phased over a defined period (possibly seperated by hours or even days). Preferably, the signals will be delivered substantially simultaneously.

Assays for monitoring expression of the one or more target genes and other methods of detecting modulation of Notch signalling are described below.

Assays

The assay of the present invention is set up to detect either inhibition or enhancement of Notch signalling in cells of the immune system by candidate modulators. The method comprises mixing cells of the immune system, where necessary transformed or transfected, etc. with a synthetic reporter gene, in an appropriate buffer, with a sufficient amount of candidate modulator and monitoring Notch signalling. The modulators may be small molecules, proteins, antibodies or other ligands as described above. Amounts or activity of the target gene (also described above) will be measured for each compound tested using standard assay techniques and appropriate controls. Preferably the detected signal is compared with a reference signal and any modulation with respect to the reference signal measured.

The assay may also be run in the presence of a known antagonist of the Notch signalling pathway in order to identify compounds capable of rescuing the Notch signal.

Any one or more of appropriate targets - such as an amino acid sequence and/or nucleotide sequence - may be used for identifying a compound capable of modulating the Notch signalling pathway in cells of the immune system in any of a variety of drug screening techniques. The target employed in such a test may be free in solution,

affixed to a solid support, borne on a cell surface, or located intracellularly. The assay of the present invention is a cell based assay.

The assay of the present invention may be a screen, whereby a number of agents are tested. In one aspect, the assay method of the present invention is a high through put screen.

Techniques for drug screening may be based on the method described in Geysen, European Patent No. 0138855, published on September 13, 1984. In summary, large numbers of different small peptide candidate modulators are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable target or fragment thereof and washed. Bound entities are then detected - such as by appropriately adapting methods well known in the art. A purified target can also be coated directly onto plates for use in drug screening techniques. Plates of use for high throughput screening (HTS) will be multi-well plates, preferably having 96, 384 or over 384 wells/plate. Cells can also be spread as "lawns". Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support. High throughput screening, as described above for synthetic compounds, can also be used for identifying organic candidate modulators.

This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a target specifically compete with a test compound for binding to a target.

It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

Various nucleic acid assays are also known. Any conventional technique which is known or which is subsequently disclosed may be employed. Examples of suitable nucleic acid assay are mentioned below and include amplification, PCR, RT-PCR, RNase protection, blotting, spectrometry, reporter gene assays, gene chip arrays and other hybridization methods.

Target gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of target mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe. Those skilled in the art will readily envisage how these methods may be modified, if desired.

PCR was originally developed as a means of amplifying DNA from an impure sample. The technique is based on a temperature cycle which repeatedly heats and cools the reaction solution allowing primers to anneal to target sequences and extension of those primers for the formation of duplicate daughter strands. RT-PCR uses an RNA template for generation of a first strand cDNA with a reverse transcriptase. The cDNA is then amplified according to standard PCR protocol. Repeated cycles of synthesis and denaturation result in an exponential increase in the number of copies of the target DNA produced. However, as reaction components become limiting, the rate of amplification decreases until a plateau is reached and there is little or no net increase in PCR product. The higher the starting copy number of the nucleic acid target, the sooner this "end-point" is reached.

Real-time PCR uses probes labeled with a fluorescent tag or fluorescent dyes and differs from end-point PCR for quantitative assays in that it is used to detect PCR products as they accumulate rather than for the measurement of product accumulation after a fixed number of cycles. The reactions are characterized by the point in time during cycling when amplification of a target sequence is first detected through a significant increase in fluorescence.

The ribonuclease protection (RNase protection) assay is an extremely sensitive technique for the quantitation of specific RNAs in solution . The ribonuclease protection assay can be performed on total cellular RNA or poly(A)-selected mRNA as a target. The sensitivity of the ribonuclease protection assay derives from the use of a complementary *in vitro* transcript probe which is radiolabeled to high specific activity. The probe and target RNA are hybridized in solution, after which the mixture is diluted and treated with ribonuclease (RNase) to degrade all remaining single-stranded

RNA. The hybridized portion of the probe will be protected from digestion and can be visualized via electrophoresis of the mixture on a denaturing polyacrylamide gel followed by autoradiography. Since the protected fragments are analyzed by high resolution polyacrylamide gel electrophoresis, the ribonuclease protection assay can be employed to accurately map mRNA features. If the probe is hybridized at a molar excess with respect to the target RNA, then the resulting signal will be directly proportional to the amount of complementary RNA in the sample.

PCR technology as described e.g. in section 14 of Sambrook et al., 1989, requires the use of oligonucleotide probes that will hybridise to target nucleic acid sequences. Strategies for selection of oligonucleotides are described below.

As used herein, a probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most preferably at least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases. The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of polypeptides. The nucleic acids used as probes may be degenerate at one or more positions.

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labelled with suitable label means for ready detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labelling a DNA fragment is by incorporating α^{32}P dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labelled with γ^{32}P-labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g. enzyme labelling, fluorescent labelling with suitable fluorophores and biotinylation.

Preferred are such sequences, probes which hybridise under high-stringency conditions.

Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (Tm) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M Na+ at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na+ pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, et al., eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York or Ausubel, et al., eds. (1990) Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the hybridising pair also play a role.

Gene expression may also be detected using a reporter system. Such a reporter system may comprise a readily identifiable marker under the control of an expression system, e.g. of the gene being monitored. Fluorescent markers, which can be detected and sorted by FACS, are preferred. Especially preferred are GFP and luciferase. Another type of preferred reporter is cell surface markers, i.e. proteins expressed on the cell surface and therefor easily identifiable.

In general, reporter constructs useful for detecting Notch signalling by expression of a reporter gene may be constructed according to the general teaching of Sambrook et al (1989). Typically, constructs according to the invention comprise a promoter of the gene of interest (i.e. of an endogenous target gene), and a coding sequence encoding the desired reporter constructs, for example of GFP or luciferase. Vectors encoding GFP and luciferase are known in the art and available commercially.

Sorting of cells, based upon detection of expression of target genes, may be performed by any technique known in the art, as exemplified above. For example, cells may be sorted by flow cytometry or FACS. For a general reference, see Flow Cytometry and Cell Sorting: A Laboratory Manual (1992) A. Radbruch (Ed.), Springer Laboratory, New York.

Flow cytometry is a powerful method for studying and purifying cells. It has found wide application, particularly in immunology and cell biology: however, the capabilities of the FACS can be applied in many other fields of biology. The acronym F.A.C.S. stands for Fluorescence Activated Cell Sorting, and is used interchangeably with "flow cytometry". The principle of FACS is that individual cells, held in a thin stream of fluid, are passed through one or more laser beams, causing light to be scattered and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) convert light to electrical signals, which are interpreted by software to generate data about the cells. Sub-populations of cells with defined characteristics can be identified and automatically sorted from the suspension at very high purity (~100%).

FACS can be used to measure target gene expression in cells transfected with recombinant DNA encoding polypeptides. This can be achieved directly, by labelling of the protein product, or indirectly by using a reporter gene in the construct. Examples of reporter genes are β-galactosidase and Green Fluorescent Protein (GFP). β-galactosidase activity can be detected by FACS using fluorogenic substrates such as fluorescein digalactoside (FDG). FDG is introduced into cells by hypotonic shock, and is cleaved by the enzyme to generate a fluorescent product, which is trapped within the cell. One enzyme can therefor generate a large amount of fluorescent product. Cells expressing GFP constructs will fluoresce without the addition of a substrate. Mutants of GFP are available which have different excitation frequencies, but which emit fluorescence in the same channel. In a two-laser FACS machine, it is possible to distinguish cells which are excited by the different lasers and therefor assay two transfections at the same time.

Alternative means of cell sorting may also be employed. For example, the invention comprises the use of nucleic acid probes complementary to mRNA. Such probes can be used to identify cells expressing polypeptides individually, such that they may subsequently be sorted either manually, or using FACS sorting. Nucleic acid probes complementary to mRNA may be prepared according to the teaching set forth above, using the general procedures as described by Sambrook et al (1989).

In a preferred embodiment, the invention comprises the use of an antisense nucleic acid molecule, complementary to a target mRNA, conjugated to a fluorophore which may be used in FACS cell sorting.

Methods have also been described for obtaining information about gene expression and identity using so-called gene chip arrays or high density DNA arrays (Chee). These high density arrays are particularly useful for diagnostic and prognostic purposes. Use may also be made of In Vivo Expression Technology (IVET) (Camilli). IVET identifies target genes up-regulated during say treatment or disease when compared to laboratory culture.

The advantage of using a protein assay is that Notch activation can be directly

measured. Assay techniques that can be used to determine levels of a polypeptide are well known to those skilled in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis, antibody sandwich assays, antibody detection, FACS and ELISA assays.

The invention, in certain embodiments, includes antibodies specifically recognising and binding to polypeptides.

Antibodies may be recovered from the serum of immunised animals. Monoclonal antibodies may be prepared from cells from immunised animals in the conventional manner.

The antibodies of the invention are useful for identifying cells expressing the genes being monitored.

Antibodies according to the invention may be whole antibodies of natural classes, such as IgE and IgM antibodies, but are preferably IgG antibodies. Moreover, the invention includes antibody fragments, such as Fab, F(ab')2, Fv and ScFv. Small fragments, such Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.

The antibodies may comprise a label. Especially preferred are labels which allow the imaging of the antibody in neural cells in vivo. Such labels may be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within tissues. Moreover, they may be fluorescent labels or other labels which are visualisable in tissues and which may be used for cell sorting.

For assays involving monitoring or detection of tolerised T-cells for use in clinical applications, the assay will generally involve removal of a sample from a patient prior to the step of detecting a signal resulting from cleavage of the intracellular domain.

The invention additionally provides a method of screening for a candidate modulator of Notch signalling, the method comprising mixing in a buffer an appropriate amount of Notch, wherein Notch is suitably labelled with detection means for monitoring cleavage of Notch; and a sample of a candidate ligand; and monitoring any cleavage of Notch.

As used herein, the term "sample" refers to a collection of inorganic, organic or biochemical molecules which is either found in nature (e.g., in a biological- or other specimen) or in an artificially-constructed grouping, such as agents which may be found and/or mixed in a laboratory. The biological sample may refer to a whole organism, but more usually to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, saliva and urine).

The present invention provides a method of detecting novel modulators of Notch signalling. The modulators identified may be used as therapeutic agents – i.e. in therapy applications.

Therapy

The term "therapy" includes curative effects, alleviation effects, and prophylactic effects. The therapy may be on humans or animals.

Modulators identified by the assay method of the present invention may be used to treat disorders and/or conditions of the immune system. In particular, the compounds can be used in the treatment of T cell mediated diseases or disorders. A detailed description of the conditions affected by the Notch signalling pathway may be found in our WO98/20142, WO00/36089 and WO/00135990.

Diseased or infectious states that may be described as being mediated by T cells include, but are not limited to, any one or more of asthma, allergy, tumour induced aberrations to the T cell system and infectious diseases such as those caused by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas,

Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara. Thus particular conditions that may be treated or prevented which are mediated by T cells include multiple sclerosis, rheumatoid arthritis and diabetes. The present invention may also be used in organ transplantation or bone marrow transplantation. The present invention is also useful in treating immune disorders such as autoimmune disorders or graft rejection such as allograft rejection.

Examples of autoimmune disorders range from organ specific diseases (such as thyroiditis, insulitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis) to systemic illnesses such as rheumatoid arthritis or lupus erythematosus. Other disorders include immune hyperreactivity, such as allergic reactions.

In more detail, organ-specific autoimmune diseases include multiple sclerosis, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic), autoimmune hepatitis, thyroiditis, insulitis, iridocyclitis, skleritis, uveitis, orchitis, myasthenia gravis, idiopathic thrombocytopenic purpura, inflammatory bowel diseases (Crohn's disease, ulcerative colitis).

Systemic autoimmune diseases include: rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, sjogren's syndrom, undifferentiated connective tissue syndrome, antiphospholipid syndrome, different forms of vasculitis (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essentiell (mixed) cryoglobulinemia, Psoriasis vulgaris and psoriatic arthritis, diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatoid granulomatosis, erythema nodosum, ankylosing spondylitis, Reiter's syndrome, different forms of inflammatory dermatitis.

A more extensive list of disorders includes: unwanted immune reactions and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome,

Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery or organ, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

The present invention is also useful in cancer therapy, particularly in diseases involving the conversion of epithelial cells to cancer. The present invention is especially useful in relation to adenocarcinomas such as: small cell lung cancer, and cancer of the kidney, uterus, prostrate, bladder, ovary, colon and breast.

Pharmaceutical Compositions

The present invention provides a pharmaceutical composition comprising administering a therapeutically effective amount of at least one compound identified by the method of the present invention and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit.

1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Where the compound is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile

aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

Administration

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The compositions of the present invention may be administered by direct injection. The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectos, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral, gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestable solution; a parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular, intradermal, intra-articular, intrathecal, intra-peritoneal or subcutaneous route, or via the alimentary tract (for example, via the Peyers patches).

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient. Preferably the pharmaceutical compositions are in unit dosage form. The present invention includes both human and veterinary applications.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

References

Tamura et al. (1995) Curr. Biol. **5**:1416-1423.
Artavanis-Tsakomas et al. (1995) Science **268**:225-232.
Artavanis-Tsakomas et al. (1999) Science **284**:770-776.
Lieber et al. (1993) Genes Dev **7**(10):1949-65.
Schroeter et al. (1998) Nature **393**(6683):382-6.
Struhl et al. (1998) Cell **93**(4):649-60.
Weinmaster(2000) Curr. Opin. Genet. Dev. **10**:363-369.
Lu et al. (1996) Proc Natl Acad Sci **93**(11):5663-7.
Munro and Freeman (2000) Curr. Biol. **10**:813-820.
Ju et al. (2000) Nature **405**:191-195.
Moloney et al. (2000) Nature **406**:369-375.
Brucker et al. (2000) Nature **406**:411-415.
Panin et al. (1997) Nature **387**:908-912.

Hicks et al. (2000) Nat. Cell. Biol. **2**:515-520.

Irvine (1999) Curr. Opin. Genet. Devel. **9**:434-441.

Devereux et al. (1984) Nucleic Acid Research **12**:87.

Atschul et al. (1990) J. Mol. Biol. 403-410.

Inaba et al. (1992) J. Exp. Med. **175**:1157-1167.

Caux et al. (1992) Nature **360**:258-261.

Coffin et al. (1998) Gene Therapy **5**:718-722.

Kroll et al. (1993) DNA Cell Biol. **12**:441-453.

Osborne and Miele (1999) Immunity **11**:653-663.

Matsuno et al. (1995) Development **121**(8):2633-44.

Matsuno et al. (1998) Nat. Genet. **19**:74-78.

Ordentlich et al. (1998) Mol. Cell. Biol. **18**:2230-2239.

Takebayashi et al. (1994) J Biol Chem **269**(7):150-6.

Leimeister et al. (1999) Mech Dev **85**(1-2):173-7.

Maddox (1983) J. Exp. Med. **15**(8):121 1

Sauber et al (1995) Virol. **213**:439-449

Chee et al. (1996) Science **274**:601-614.

Camilli et al. (1994) Proc Natl Acad Sci USA **91**:2634-2638.

Hoyne et al. (2000) Immunology **100**:281-288.

Hoyne et al. (2001) – reference in press.

CLAIMS

1. A method for detecting modulators of Notch signalling comprising the steps of monitoring Notch signalling in a cell of the immune system in the presence and absence of a candidate modulator, and determining whether the candidate modulator modulates Notch signalling.

2. A method for detecting modulators of Notch signalling comprising the steps of:

(a) contacting a cell of the immune system with a candidate modulator;

(b) monitoring Notch signalling; and

(c) determining whether the candidate modulator modulates Notch signalling.

3. A method according to claim 1 or claim 2 wherein the candidate modulator is selected from the group consisting of: an organic compound, a inorganic compound, a peptide or polypeptide, a polynucleotide, an antibody, a fragment of an antibody, a cytokine and a fragment of a cytokine.

4. A method according to any preceding claim wherein the step of monitoring Notch signalling comprises the step of monitoring levels of expression of at least one target gene.

5. A method according to claim 4 wherein the at least one target gene is an endogenous target gene of Notch signalling.

6. A method according to claim 5 wherein the at least one target gene is selected from the group consisting of: Deltex, Hes-1, E(spl), Il-10, CD-23, Dlx-1, CTLA4, CD-4, Numb, Mastermind and Dsh.

7. A method according to claim 4 wherein the at least one target gene is a reporter gene.

8. A method according to claim 7 wherein the at least one target gene is selected from the group consisting of: a gene encoding a polypeptide having an enzymatic activity, a gene comprising a radiolabel or a fluorescent label and a gene encoding a predetermined polypeptide epitope.

9. A method according to any one of claims 4 to 8 wherein the at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling.

10. A method according to any of claims 1 to 8 wherein the at least one target gene is under the transcriptional control of a promoter region sensitive to:

i) Notch signalling; and

ii) a second signal; and/or

iii) a third signal

wherein the second and third signals are different.

11. A method according to claim 10 wherein the second signal results from activation of a signalling pathway specific to cells of the immune system.

12. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a T cell receptor (TCR) signalling pathway.

13. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a B cell receptor (BCR) signalling pathway.

14. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a Toll-like receptor (TLR) signalling pathway.

15. A method according to any one of claims 10 to 14 wherein the third signal is a costimulus specific to cells of the immune system.

16. A method according to claim 15 wherein the costimulus is selected from the group consisting of: B7 proteins B7.1-CD80, B7.2-CD86, B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD28, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134, CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205, CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLRs), such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FCγ receptor 2 (CD32), CD64 (FCγ receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors and growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.

17. A method according to any preceding claim wherein the cell of the immune system is a T cell or T cell progenitor.

18. A method according to any one of claims 1 to 16 wherein the cell of the immune system is an antigen presenting cell (APC).

19. A method according to any one of claims 4 to 18 wherein expression of the at least one target gene is monitored with a protein assay.

20. A method according to any of claims 4 to 18 wherein expression of the at least one target gene is monitored with a nucleic acid assay.

21. A modulator identifiable by a method according to any one of the preceding claims.

22. Use of a modulator according to claim 21 for the preparation of a medicament for the treatment of a disease or condition of, or related to, the immune system.

23. Use of a modulator according to claim 22 wherein the disease is a T-cell mediated disease.

24. Use of a modulator according to claim 22 wherein the disease is a B-cell mediated disease.

25. Use of a modulator according to claim 22 wherein the disease is an APC mediated disease.

26. A pharmaceutical composition comprising a therapeutically effective amount of at least one modulator according to claim 22 and a pharmaceutically acceptable carrier, diluent and/or excipient.

27. A method for detecting modulators of Notch signalling substantially as hereinbefore described with reference to the accompanying figures.

28. A modulator substantially as hereinbefore described with reference to the accompanying figures.

29. Use of a modulator substantially as hereinbefore described with reference to the accompanying figures.

30. A pharmaceutical composition substantially as hereinbefore described with reference to the accompanying figures.

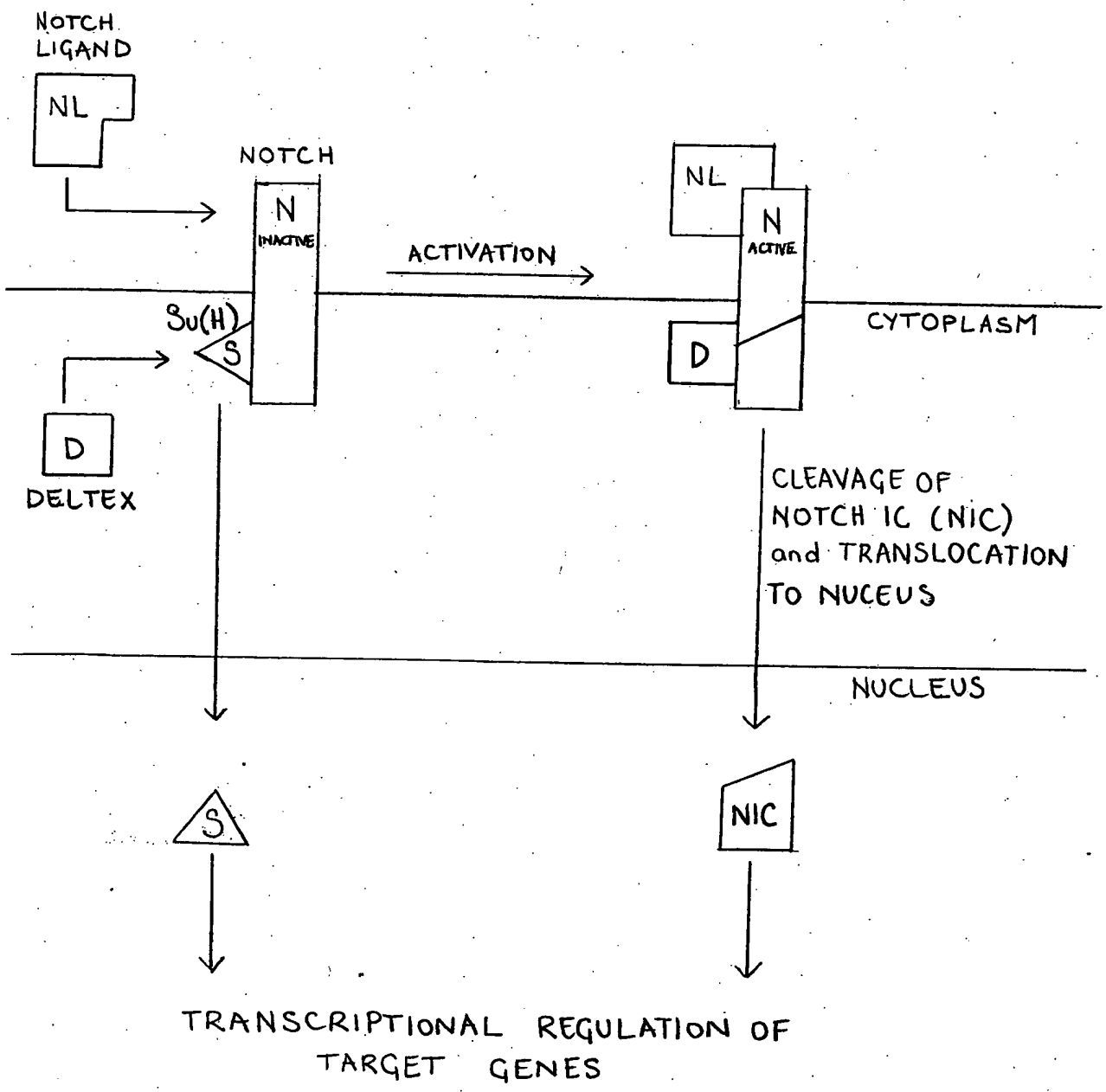
~~METHOD OF~~ MODULATORS OF NOTCH SIGNALLING

ABSTRACT

~~A METHOD~~

A method for detecting modulators of Notch signalling comprising the step of monitoring Notch signalling in a cell of the immune system in the presence of a candidate modulator. The step of monitoring may comprise the step of monitoring levels of expression of at leash one of: Deltex, Hes-1, E(Spl) I1-10, CD-23, Dlx-1, CTLA4, CD-4, Numb, Mastermind and Dsh.

FIGURE 1


NOTCH LIGAND
NL
NOTCH
N
INACTIVE
ACTIVATION
NL
N
ACTIVE
Su(H)
S
CYTOPLASM
D
D
DELTEX
CLEAVAGE OF NOTCH IC (NIC) and TRANSLOCATION TO NUCEUS
NUCLEUS
S
NIC
TRANSCRIPTIONAL REGULATION OF TARGET GENES

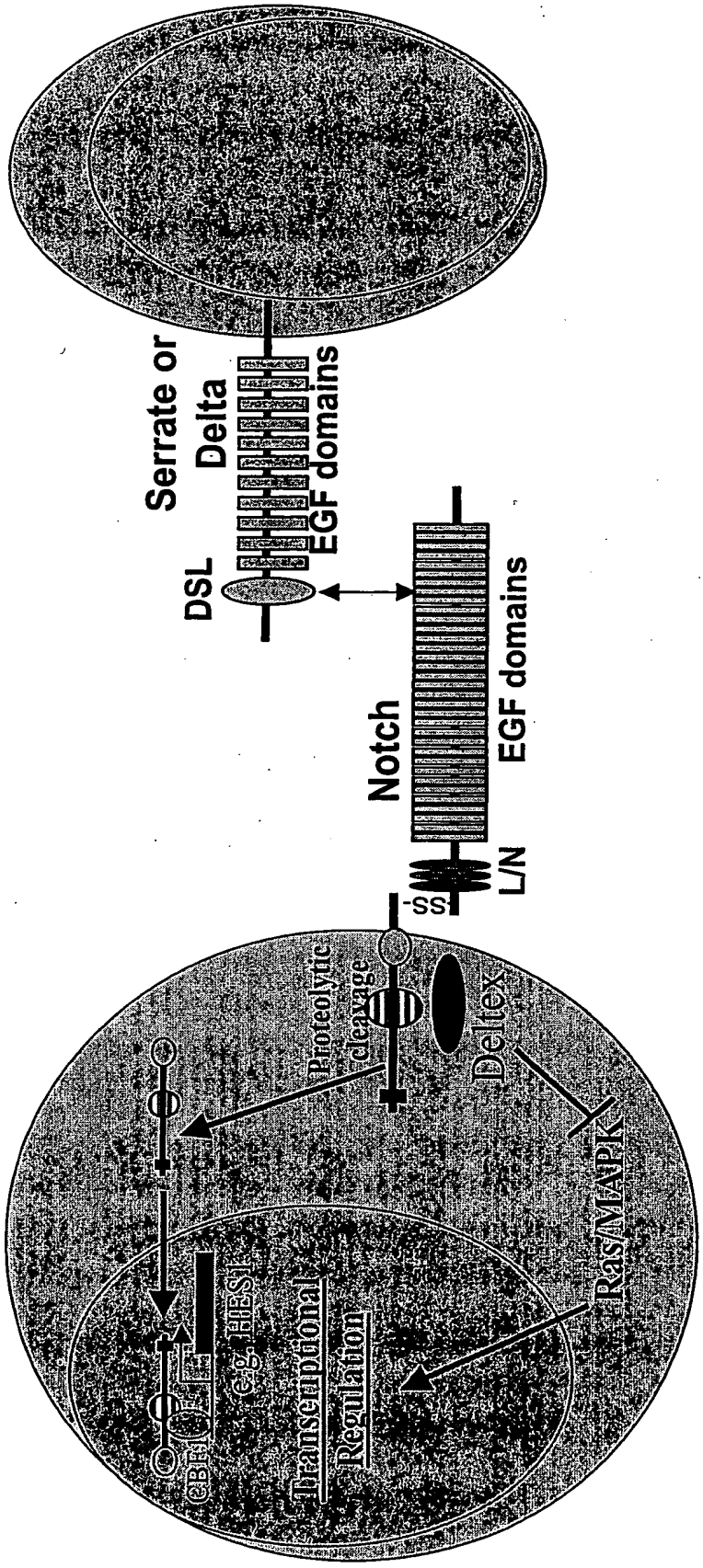
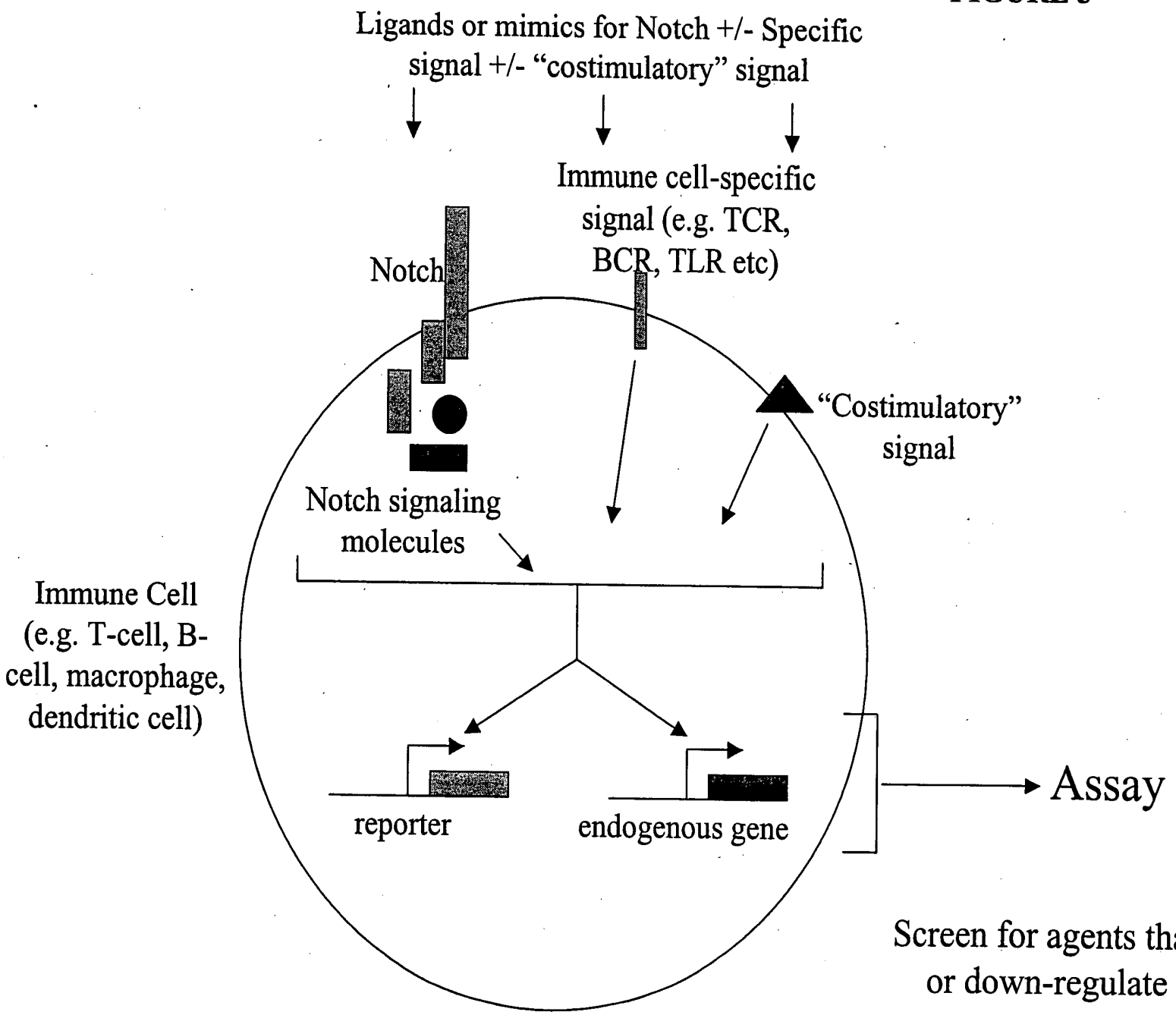

Serrate or
Delta
DSL
EGF domains
Notch
EGF domains
L/N
-SS-
Proteolytic
cleavage
Deltex
Ras/MAPK
CBF1/E
Transcriptional
Regulation

Figure 2

FIGURE 3


Ligands or mimics for Notch +/- Specific signal +/- "costimulatory" signal
Immune cell-specific signal (e.g. TCR, BCR, TLR etc)
Notch
"Costimulatory" signal
Notch signaling molecules
Immune Cell (e.g. T-cell, B-cell, macrophage, dendritic cell)
reporter
endogenous gene
Assay
Screen for agents that up- or down-regulate the response